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REACTIONS OF A GLUCOSINOLATE BREAKDOWN PRODUCT (BENZYL ISOTHIOCYANATE) WITH MYOGLOBIN

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Key Word Index—Glucosinolate; breakdown products; benzyl-isothiocyanate; protein derivatization; myoglobin; lysine reaction; thiourea formation; physico-chemical characterisation; MALDI-TOF-MS.

Abstract—The interaction of various amounts of benzyl isothiocyanate (benzyl-ITC) with myoglobin is known to lead to the formation of derivatives. These have been characterised by the determination of solubility, free amino group, tryptophan content and chromatographic as well as electrophoretic behaviour. In the range between 2.5 and 125 mg benzyl-ITC/g protein, all properties of the reaction products correlate with the concentration of benzyl-ITC. However, at 250 mg benzyl-ITC/g myoglobin, a rather unexpected low degree of derivatization, as well as atypical chromatographic and electrophoretic behaviour, is observed. The proposed explanation was that conformational changes in the presence of a high concentration of hydrophobic benzyl-ITC made fewer amino groups accessible to the reagent. To test this hypothesis we have run the reaction under denaturing conditions. The results showed that the reaction of myoglobin with high concentrations of benzyl-ITC in the presence of 8 M urea led to a higher degree of derivatization than in the presence of water only. In addition, the Mr distribution of the reaction products was determined by MALDI-TOF-mass spectrometry and the overall degree of derivatization calculated from the spectra.

INTRODUCTION

Several isothiocyanates (ITC) are nutritionally important products arising from hydrolysis and rearrangement of glucosinolates which are widely distributed in Cruciferae, such as cabbage, cauliflower and Brussels sprouts [1]. ITC reacts with free amino and sulphydryl groups of various proteins [2–5]. It was shown recently that the reaction of benzyl-ITC with myoglobin—which is devoid of thiol groups—leads also to modification of Trp residues [6]. While some properties of the reaction products, such as solubility, free amino group, Trp content, retention volume on reverse phase HPLC, pI as determined by IEF and electrophoretic behaviour in the PAGE and SDS-PAGE, correlate with the concentration of benzyl-ITC in the range between 2.5 and 125 mg benzyl-ITC/g of protein, a rather unexpected low degree of derivatization, as well as atypical chromatographic and electrophoretic behaviour, was found in the product obtained from the reaction with 250 mg benzyl-ITC/g myoglobin [6]. It was assumed that in this case, fewer amino groups were accessible to the reagent because the protein chain is more compact in the presence of a high concentration of the hydrophobic benzyl-ITC. An experimental approach for testing this hypothesis would be to run the reaction in the presence of a denaturation agent. In this paper, we report new data on the reaction of benzyl-ITC at high concentration in the presence of 8 M urea, including also the determination of the degree of derivatization by MALDI-TOF-mass spectrometry.

RESULTS AND DISCUSSION

Solubility

As reported previously [6], the solubility behaviour of a derivative of myoglobin prepared in the presence of 250 mg benzyl-ITC/g protein is virtually indistinguishable from that of native myoglobin. However, a dramatic decrease in solubility ensues when the reaction with 250 mg benzyl-ITC/g protein is carried out

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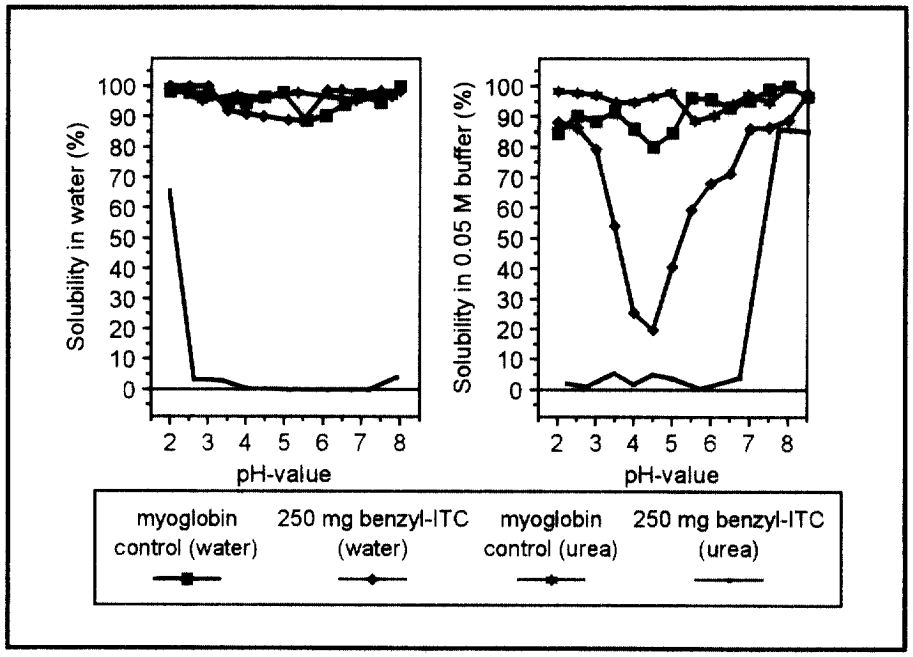

Fig. 1. Solubility of derivatives of benzyl-ITC and myoglobin in water or in phosphate buffer.

in 8 M urea (Fig. 1) This latter material is poorly soluble at pH > 2. When the ionic strength was increased by using 50 mM sodium phosphate buffer, the sample prepared in water showed a solubility minimum at pH 4.5. In contrast, the solubility of the derivative prepared in 8 M urea solution was low at pH < 7, but, due to the, "salting-in" effect, increased by almost 85% when the pH was raised to 7.7. The solubility of the 250 mg benzyl-ITC/g protein derivative prepared in 8 M urea solution was even lower than that of a sample prepared with 125 mg benzyl-ITC/g protein sample in water [6]. Thus, at a higher degree of derivatization there was a profound decrease in protein solubility over a wide pH range, as was also observed for egg-white proteins [3, 4]. This result reflects the fact that in the reaction of the protein with benzyl-ITC, charged amino groups in the protein are replaced by hydrophobic benzyl thiourea groups making the derivative less soluble in polar solvents like water.

Free amino group content

The reaction with 250 mg benzyl-ITC/g protein in water led to a decrease of ca 27% of free amino groups as compared with ca 44% when the reaction was carried out in 8 M urea (Table 1). This latter value was similar to the degree of derivatization with 125 mg benzyl-ITC/g protein in water [6]. This indicates that even under denaturing conditions, about half of the amino groups in myoglobin are still shielded and do not react with benzyl-ITC. Surprisingly, in sample 4 fewer free amino groups (and thus a higher degree of derivatization in samples 4 and 5, see Table 2) were

Table 1. Characterisation of the derivatives from myoglobin and benzyl-ITC

	Free amino groups (nmol/mg protein)/(% of the total)			
	In relation to total proteins*		In relation to water-soluble proteins	
Sample/derivative	nmol/mg	%	nmol/mg	%
(1) Myoglobin in water	1244.8 ± 8.8	100	1261.5 ± 17.7	100
(2) Myoglobin in 8 M urea	1243.5 ± 8.7	100	1272.2 ± 3.3	100
(3) Myoglobin + 250 mg benzyl-ITC/g protein in water	910.1 ± 30.0	73	1007.4 ± 17.0	80
(4) Myoglobin + 250 mg benzyl-ITC/g protein in 8 M urea	701.6 ± 9.7	56	547.8 ± 2.3	43

*Soluble in 1% SDS solution.

Table 2. Overall degrees of derivatization calculated from the MALDI-TOF mass spectra and from the results of the free amino group determinations (see Table 1)

Sample/derivative	Overall degree of derivatization determined from MALDI-TOF mass spectra	Overall degree of derivatization determined from free amino groups	
		*	**
(1) Myoglobin in water	0%	0%	0%
(3) Myoglobin + 250 mg benzyl-ITC/g protein in water	18%	27%	20%
(4) Myoglobin + 250 mg benzyl-ITC/g protein in 8 M urea	61%	44%	57%
(5) Myoglobin + 125 mg benzyl-ITC/g protein in water	46%	49%†	55%†

*In relation to total protein, soluble in 1% SDS solution. **In relation to water-soluble proteins. †Obtained from [6].

determined for the water-soluble proteins as compared to total proteins (Table 1). These results are unexpected and further investigations are necessary to explain this phenomenon.

PAGE

The electrophoretic mobility of the protein towards the positive electrode is expected to increase if the overall charge of the protein becomes more negative due to reaction of basic amino groups with benzyl-ITC. Indeed, PAGE performed under denaturing conditions (in presence of 8 M urea) showed profound differences in the separation pattern of the products prepared with 250 mg benzyl-ITC/g in water or in 8 M urea (Fig. 2). The electropherogram of the derivative prepared in water displayed five subfractions, and a higher electrophoretic mobility than the control protein. The preparation obtained from the reaction in 8 M urea presented a more complicated electrophoretic pattern. A fraction of the protein showed a very high electrophoretic mobility as expected for a highly derivatized protein. Similar electrophoretic mobilities were previously observed for a sample derivatized with 125 mg benzyl-ITC/g protein [6]. Surprisingly, a large amount of protein showed an extremely low mobility, i.e., the material remained at the start of the separating gel. This effect could be explained by the presence of aggregated material due to the low solubility of the myoglobin derivative. A third fraction of the protein, representing ca 10–20% of the total protein amount, displayed an intermediate mobility similar to that observed for the derivative prepared in water. The origin of this effect remains speculative but could be due to the presence of incompletely denatured protein.

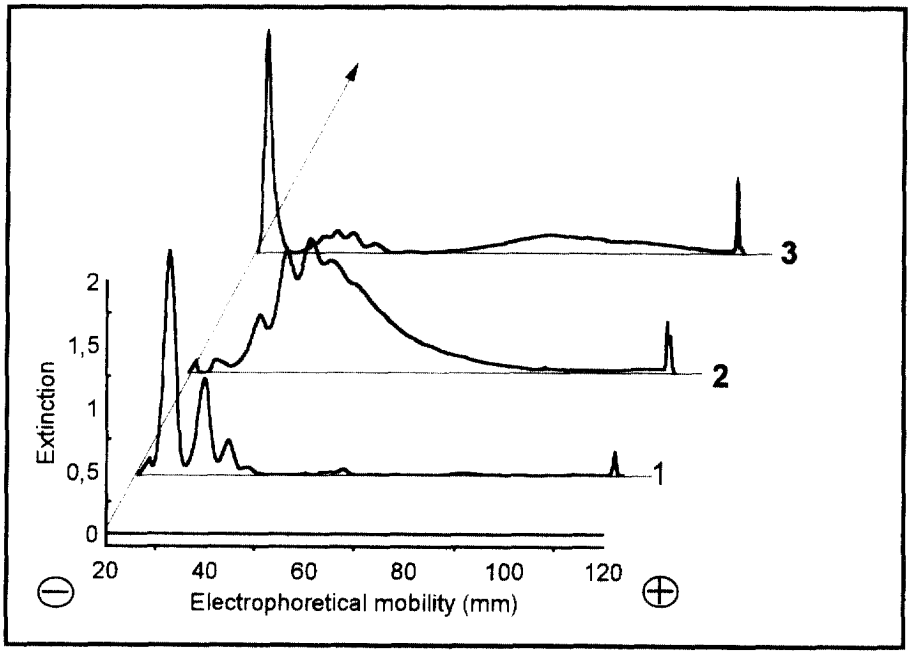

Fig. 2. PAGE of myoglobin and its derivatives in the presence of 8 M urea. 1, myoglobin, control; 2, 250 mg benzyl-ITC/g protein–water; 3, 250 mg benzyl-ITC/g protein–urea.

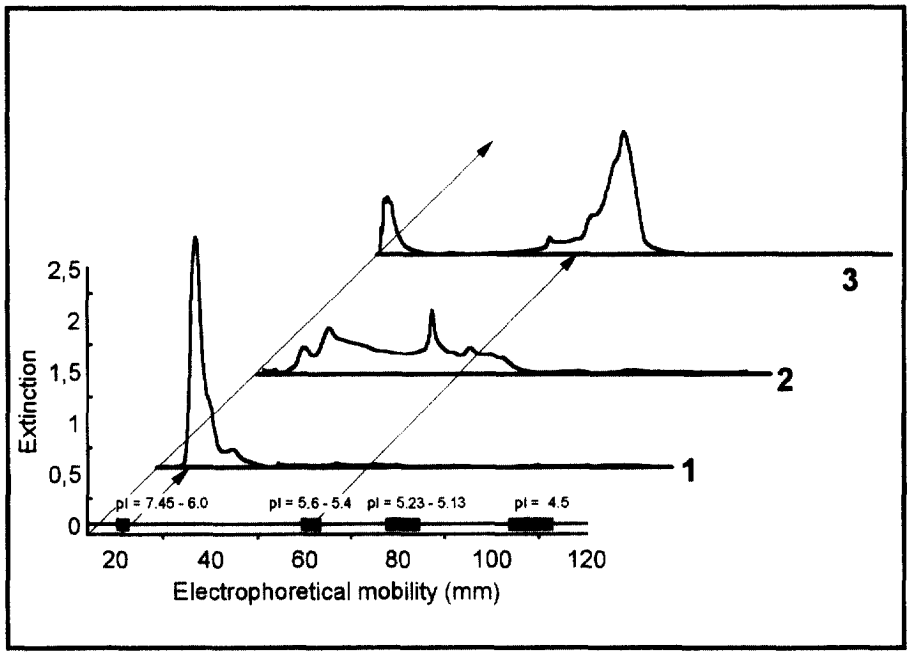

Fig. 3. IEF of myoglobin and its derivatives with benzyl-ITC. For 1–3 see legend to Fig. 2.

IEF

The sample prepared with 250 mg benzyl-ITC/g myoglobin in water showed no clear separation in IEF, indicating the presence of many molecular species with pI values between 5.3 and 7 (Fig. 3). Reaction with 250 mg benzyl-ITC in 8 M urea leads to a different separation pattern in which most of the components have pI values between 5.4 and 5.6, which is consistent with a higher loss of basic groups in this case. This is consistent with the higher electrophoretic mobility observed by PAGE.

Reverse phase HPLC

The hydrophobicity of the derivatives should increase with a higher degree of derivatization, due to the loss of charge by blocking of the free amino groups and addition of aromatic residues in the form of benzyl thiourea derivatives. However, the sample prepared with 250 mg benzyl-ITC in water is less hydrophobic than that obtained from 125 mg benzyl-ITC/g [6]. In contrast, derivatization of myoglobin with 250 mg benzyl-ITC/g of protein in the presence of 8 M urea, leads to a product that is even more hydrophobic than the 125 mg benzyl-ITC/g protein sample (Fig. 4) [6]. These results are in excellent agreement with the solubility measurements presented above.

SDS-PAGE

SDS-PAGE reveals increasing amounts of high Mr species, possibly dimers, with increasing derivatization [6]. An exception here was again the 250 mg benzyl-ITC/g protein derivative prepared in water. However, when the derivatization was preformed with 250 mg benzyl-ITC in 8 M urea solution, a higher content of dimers was observed than in the sample prepared in water (Fig. 5). In addition, two fractions with Mr > 100,000, accounting for ca 2% of the total amount, were detected. Accordingly, the content of the main fraction of myoglobin derivatives decreased to 83%, as compared with 86% in the 125 mg benzyl-ITC/g derivative [1]. The question about the origin and the nature (covalent or non-covalent) of the multimers can not be answered yet. No mechanism for the formation of covalent multimers of myoglobin under the influence of benzyl-ITC has been proposed so far. One explanation could be, that the strong hydrophobic interactions between the derivatized molecules favour non-covalent di- or multimer formation and that this interaction is stable even in a highly denaturing environment.

MALDI-TOF-MS

The MALDI mass spectra of the samples obtained from the reaction of different amounts of benzyl-ITC with myoglobin (between 2.5 and 250 mg/g protein), including the sample prepared with 250 mg/g protein in 8 M urea, are depicted in Fig. 6. Each spectrum shows a mixture of products with different degrees of derivatization. The peaks are separated by an increment of 149 amu, corresponding to the addition of one molecule of benzyl-ITC. Since the method does

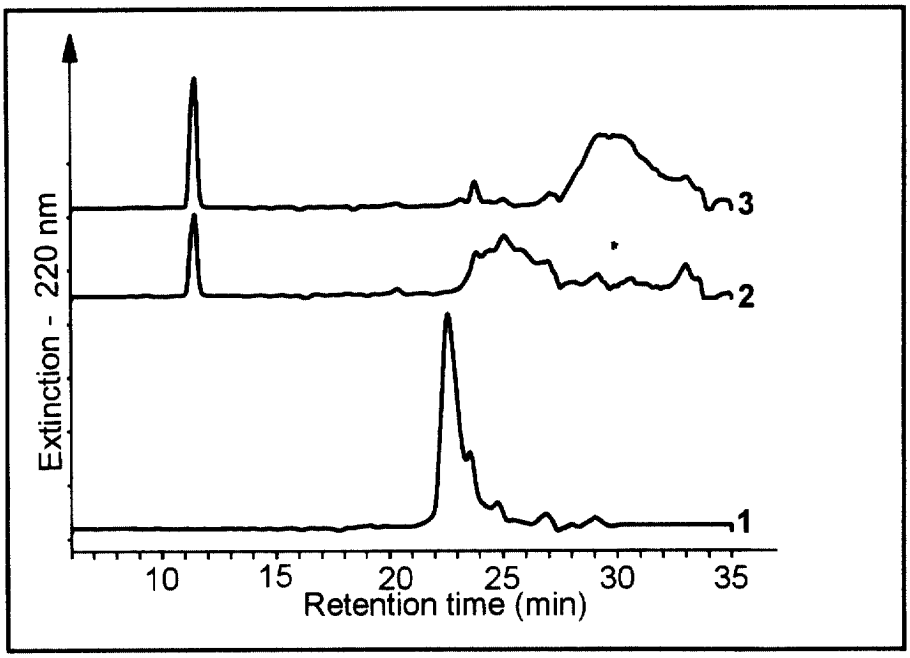

Fig. 4. RP-HPLC of derivatives from myoglobin and benzyl-ITC. For 1–3 see legend to Fig. 2.

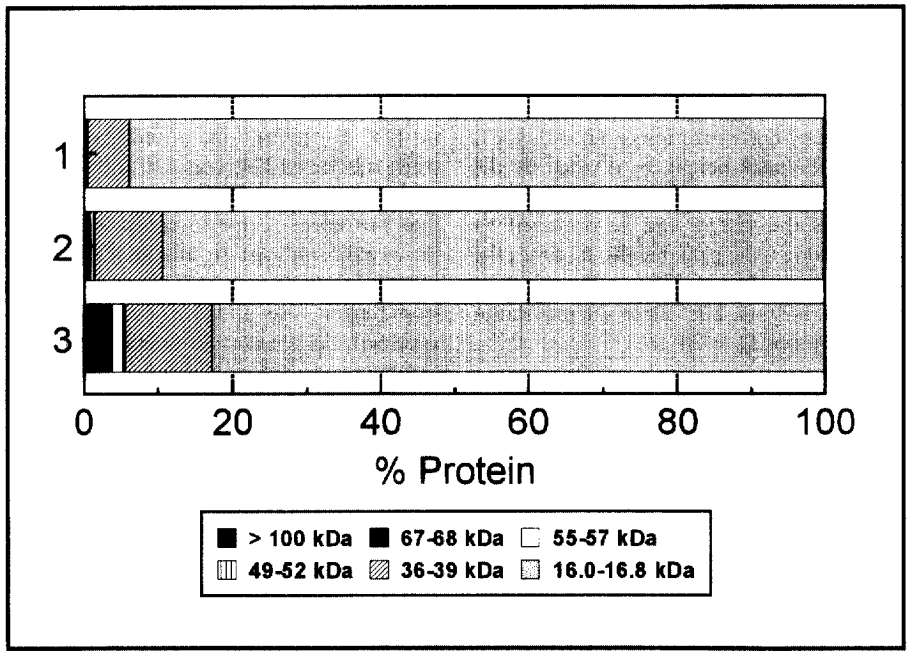

Fig. 5. SDS-PAGE of myoglobin and its derivatives with benzyl-ITC. For 1–3 see legend to Fig. 2.

not allow identification of the position of the reacting amino group, a peak of a distinct Mr certainly represents several isomers.

Horse myoglobin contains an N-terminal Gly and 19 Lys residues. Thus, the maximum possible mass is calculated to be $m/z = (16{,}951 + 20 \times 149) = 19{,}931$. The highest mass found in the case of derivatization with 125 mg benzyl-ITC/g myoglobin was m/z 19,333, which accounts for the addition of a maximum of 16 benzyl-ITC groups per molecule of myoglobin.

The overall degree of derivatization of the product mixture obtained can be calculated from the spectra.

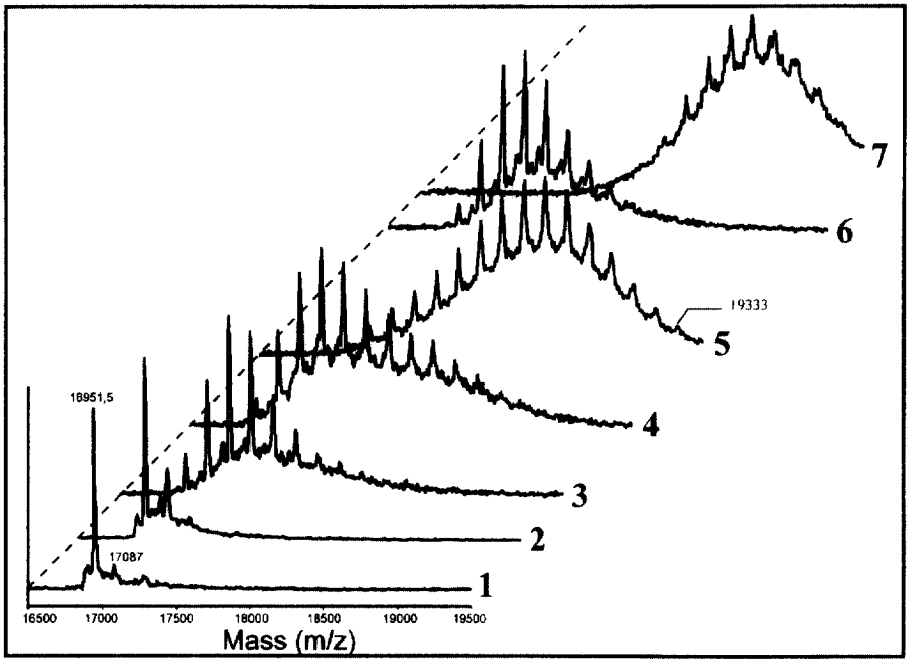

Fig. 6. MALDI-MS of myoglobin and its derivatives with benzyl-ITC. 1, myoglobin, control; 2, 2.5 mg benzyl-ITC/g protein; 3, 25 mg benzyl-ITC/g protein; 4, 62.5 mg benzyl-ITC/g protein; 5, 125 mg benzyl-ITC/g protein; 6, 250 mg benzyl-ITC/g protein–water; 7, 250 mg benzyl-ITC/g protein—urea.

Every peak, i ($i = 0, 1, ..., 20$), in a spectrum (Fig. 6), represents a certain fraction, f_i, of the protein with a well-defined degree of derivatization, I_i ($I_i = 0, 1, ..., 20$). f_i is given by the peak height, h_i, divided by the sum of all peak heights, i.e. $h_i/\Sigma h_i$. The overall degree of derivatization of the sample can be obtained by summing for all peaks, i, the peak height, h_i, times the degree of derivatization, I_i, and dividing the result by the sum of all peak heights, Σh_i, times the maximum degree of derivatization, $I_{20} = 20$:

$$\text{Overall degree of derivatization} = \frac{\Sigma_i h_i \cdot I_i}{20\,\Sigma_i h_i}$$

The results obtained are summarised in Table 2 and compared to the values calculated from the free amino groups determination (Table 1).

As indicated by the values in Table 2, the overall degree of derivatization increases in the order 250 mg benzyl-ITC in water < 125 mg in water < 250 mg in 8 M urea. Furthermore, the values calculated from the mass spectra are in good agreement with the data obtained from free amino group determination. This indicates that our working hypothesis, i.e. mainly amino groups react with benzyl-ITC, holds true. Whether the two Trp residues also react with benzyl-ITC, as was suggested by [6], can not be decided from the above results. The number of Trp residues is too low in comparison with the number of reacting amino groups.

The exceptional behaviour of the derivative prepared with 250 mg benzyl-ITC/g protein in water can clearly be seen in Fig. 6. After a steady increase of overall degree of derivatization with increasing benzyl-ITC concentration, a sudden drop is observed when the benzyl-ITC concentration is raised from 125 to 250 mg benzyl-ITC/g protein. It was postulated that this effect is due to conformational changes of the protein induced by the increasing concentration of hydrophobic benzyl-ITC. To test this, urea, a chaotropic agent, was added to the reaction mixture. As expected, the presence of urea loosened the otherwise compact conformation of the protein and allowed the reaction of more reactive groups. The reaction with 250 mg benzyl-ITC/g protein in 8 M urea resulted in the highest overall degree of derivatization observed.

EXPERIMENTAL

Myoglobin from horse heart and benzyl-ITC were purchased from Fluka. The derivatives were prepared as described [6]. An additional derivatization was done at a concentration of 250 mg benzyl-ITC/g protein in 8 M urea.

For determination of solubilities, free amino groups and tryptophan content, fluorescence measurements, RP-HPLC, IEF, SDS-PAGE, and PAGE in the presence of 8 M urea, see [6]. Since the derivatives were hardly soluble in water at pH 2.5 (pH adjusted with 5 g trifluoroacetic acid litre^{-1}), they were dispersed in 8 M urea before RP-HPLC analysis. PAGE and IEF were carried out in ca 14 cm gels in a BIO-RAD type PROTEAN II xi cell.

MALDI-TOF mass spectrum analysis was performed on a Voyager Elite (PerSeptive Biosystems, Cambridge, U.S.A.) instrument equipped with a reflector and delayed extraction [7]. The spectra were externally calibrated using horse heart myoglobin (singly and doubly charged peaks).

The intact Mrs were measured with DHBs [8] a binary matrix consisting of 9 parts 2,5-dihydroxy benzoic acid and 1 part 2-hydroxy-5-methoxy benzoic acid, both solns 25 g/l in 35% MeCN/65% 0.1% TFA. The samples were prepared by mixing 1 μl of matrix and 0.2 μl of sample on the target and allowing it to dry in air.

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